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14. ABSTRACT

Which is the molecular mechanism that leads to DNA amplification and oncogenes activation in breast cancer cells? This project aims to understand the role of estrogen in inducing re-replication, thus leading to DNA amplifications. I worked on the establishment of cancer cell line models carrying an engineered replication origin that will be tested for undergoing DNA amplification after estrogen treatment. I performed two biological replicates of a preliminary experiment done during Year-One, but I was unable to reproduce the exciting data previously obtained: Drug selection after estrogen exposure was not efficient in selecting cells with DNA amplification at the ectopic replication origin. Optimizing the working system to have multiple possibilities on the selection of cells with DNA amplification induced by estrogen receptor alpha, a new reporter construct have been built and a more efficient cell line model (U2OS/ER-alpha) have been characterized.

15. SUBJECT TERMS
Cell lines establishment and characterization, estrogen treatment

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INTRODUCTION

DNA amplification is a hallmark of human cancers that can provide proliferative advantages to malignant cells, for example through the activation of oncogenes. Moreover, DNA amplification is often correlated with disease prognosis and progression. However, mechanisms that trigger DNA amplification are not yet fully understood. This research project aims to shed some light on the involvement of replication origins in DNA amplification. Specifically, I am interested in exploring whether the steroid hormone estrogen, when bound adjacent to a replication origin, is able to induce re-replication leading to DNA amplification in breast cancer cells. In continuation with the work carried out in Year-One, I revised the working system to optimize detection of DNA amplification at an ectopic replication origin that contains a binding element for estrogen receptor alpha. The development of an efficient model system will allow investigation of the role of estrogen leading to DNA amplification. Clarification of the molecular mechanism underlying DNA amplification is potentially an important step to efficiently counteract breast cancer cells proliferation and metastasis.

BODY

Methods

Cloning. *E. coli* strain Stbl3 (Invitrogen) were used for all the cloning steps and cells were transformed by electroporation (Micropulser, BioRad). Oligonucleotides used for cloning and PCR amplifications were purchased from either Integrated DNA Technologies or Invitrogen. TagGFP2 expression plasmid was acquired from Evrogene (Moscow, Russia). Restriction enzymes, T4 DNA Ligase, DNA polymerase I-Klenow fragment, Shrimp Alkaline Phosphatase, and T4 Polynucleotide Kinase were purchased from New England Biolabs. Cloning is performed following standard protocols (www.neb.com). At each cloning step, constructs were assessed by restriction digestion and further verified by sequencing (Genewiz, Cambridge, MA). Small scale plasmid DNA is prepared using QIAprep Spin Miniprep Kit (Qiagen) following the manufacturer's instructions. High scale plasmid DNA preparations needed for human cell line transfections is performed with PureLink HiPure Plasmid Filter Purification Kit (Invitrogen) following the manufacturer's instructions.

Establishment of Recombinant MCF7/PGK-BlaGFP_Myc and MCF7/PGK-BlaGFP_Myc6xERE Cells. 3×10^5 MCF7 Flp-In cells were co-transfected with either pFRT.PGK-BlaGFP_Myc or pFRT.PGK-BlaGFP_Myc6xERE construct and the Flippase recombinase expression plasmid pOG44 at a ratio of 1:9. FugeneHD transfection reagent was used at 3:1 FugeneHD (μL): DNA (μg) ratio according to the manufacturer's instructions. Drug selection was started after 48 hours from transfection and selective media was changed every 3-4 days until single colonies were visible.

Establishment of U2OS/ER-aplha_PGK-BlaGFP_Myc6xERE Clones. For each transfection, 2×10^5 cells were co-transfected with pPB.PGK-BlaGFP_Myc6xERE and pCMVhyPBase, which is the expression plasmid for the hyperactive version of the PiggyBac transposase (Yusa *et al.*, 2011). Transfection was performed with FugeneHD DNA transfection reagent (Promega) using 3.5:1 FugeneHD (μL): DNA (μg). After 48 hours from transfection, cells were trypsinized and 1/60 plated in a new dish in complete media additioned with 4 μg/mL blasticidin. Once resistant colonies were visible, single colonies were isolated and expanded. Duplicated dishes plating 1/12 of transfected cells were stained with Crystal Violet after drug selection to determined transposition efficiency.

DNA Isolation. Cells were lysed in TNE buffer (10 mM Tris HCl pH 8.0, 100 mM NaCl, 1 mM EDTA pH 8.0), with 1% SDS and incubated overnight with 0.2 mg/mL proteinase K at 37°C. After 30 min treatment with RNase A, DNA was isolated by standard phenol/chloroform extraction.

Southern Blot. Southern blots were performed as described in Sambrook *et al*., Molecular Cloning (1989). 10 μg genomic DNA isolated from U2OS/ER-alpha pPB.PGK-BlaGFP_Myc6xERE cells was digested overnight at 37°C with 100 units HindIII (New England Biolabs) and loaded on a 1% agarose gel. The gel was sequentially soaked with gentle agitation in 0.25 HCl for 20 min, dH$_2$O for 2 min, and twice in transfer buffer (0.4 M NaOH, 1 M NaCl) for 20 min). DNA was then transferred onto a positively charged nylon membrane (Hybond XL, Amersham) by overnight capillary alkaline transfer using the transfer buffer. The membrane was subsequently washed twice in 0.5 M Tris-HCl pH 7.2, 1M NaCl for 20 min, air dried, and baked at 80°C for 2 h. The membrane was then pre-hybridized in Hybridization Buffer (0.5 M Na$_2$HPO$_4$ pH 7.2, 7% SDS, 1 mM EDTA) at 60°C for 4 h. The 1052 bp ^{32}P labeled probe (a PCR amplified fragment of the blasticidin-2A-TagGFP ORF) was synthesized using the NEBlot kit (New England Biolabs) following the manufacturer's instruction. Free ^{32}P was removed from the reaction by gel filtration (Illustra ProbeQuant G-50 Micro Columns, GE Healthcare). The membrane was hybridized overnight at 60°C with Hybridization buffer supplemented with the radioactive probe (6×10^5 cpm/mL). The membrane was washed at room temperature 5 min with 2X SSC, 0.25% SDS, twice with 2X SSC, 0.1% SDS for 20 min, twice with 1X SSC, 0.1% SDS for 20 min. Finally, the membrane was exposed to X-Ray film at -80°C with intensifying screen for 2-14 days.

RT-qPCR. Total RNA was isolated using RNeasy Kit (Qiagen) following manufacturer's instructions. cDNA was obtained from 1 μg RNA using SuperScript III (Invitrogen) following manufacturer's instructions. After cDNA quantification (picogreen, Invitrogen), 5 ng was used to perform real time PCR. Each sample was tested in triplicate. GAPDH was used for sample normalization.

Quantitative PCR. 10 ng genomic DNA was assessed for each reaction using SYBR Green PCR Master Mix (Applied Biosystems). The amplification efficiency of each primer set was measured by testing serial dilutions of a reference sample. In each assay, samples were assessed in triplicate (expression level) or quadruplicate (DNA copy number). Assays were performed with an Applied Biosystems 7300 Real Time PCR machine.

Western Blot. Cells were washed once with ice-cold PBS and collected with a rubber spatula. Cells were then lysed in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS). After cell homogenization, insoluble material was removed by 10 min centrifugation at 13000 xg at 4°C. Total protein was determined with BCA Assay (Pierce) and 30 μg sample in 1X Laemmli buffer and 5% β-mercaptoethanol were boiled for 10 min at 95°C and loaded onto an 8% polyacrylamide gel. After semi-dry transfer, nitrocellulose filter was blocked with TBS, 5% nonfat dry milk and probed with primary antibody diluted in TBS, 3% nonfat dry milk. Detection of the fluorescent-labeled secondary antibody was performed with Li-Cor ODYSSEY CLx scanner.

FACS Analysis on Live Cells. Cells were trypsinized, washed once with ice-cold PBS and resuspended in ice-cold PBS at ~1x10^6 cells/mL. GFP fluorescence signal (ex 488 nm, em 530 nm) was measured with BD FACSCalibur Flow Cytometer performing the acquisition of at least 20x10^6 events and analysis performed with BD-CellQuest software.

Results

Task Two – Can Estrogen Drive DNA Amplification at the Ectopic c-Myc replication origin?

- **Sub-task 2c (months 13-17): Culture the engineered test (6xERE/c-Myc) and control cells (c-Myc) with estrogen and select with high doses of antibiotics those cells that have amplified the engineered c-Myc locus.**

During Year-One I performed a trial experiment in which I treated the recombinant cell line MCF7/c-Myc6xERE with 10 nM 17β-estradiol (E2) for 1 month, followed by a 15 mM G418 selection, which had been previously assessed to be the concentration of the drug required to kill cells with only 1 copy of the resistance gene placed within the construct carrying the ectopic c-Myc origin (cell with no amplification). Amplification at the ectopic replication origin will determine the presence of >1 copy of the drug resistance marker, thus making cells resistant to a higher dose of the drug. Real time PCR confirmed the presence of DNA amplification at the ectopic locus for cells that were selected this way in this preliminary experiment (Figure 1). Amplified cells have also been shown to be resistant to 20 mM and 30 mM G418. To confirm this exciting preliminary result, during Year-Two I repeated the treatment/selection process twice, using both the test MCF7/c-Myc6xERE and the control MCF7/c-Myc cell lines. As previously established, 10 nM E2 treatments were administered for 1 month, while culturing cells in phenol red-free medium with an addition of 10% charcoal/dextran treated fetal bovine serum. These particular conditions facilitated better control of the level of exposure to estrogen. In the first attempt to reproduce the data obtained during Year-One, 20 mM G418 was used to select cells with amplification at the ectopic c-Myc origin after E2 treatment. Drug selection and clone expansions took approximately 2.5 months. While few colonies were found to be resistant to 20 mM G418 for the control MCF7/c-Myc cells, no MCF7/c-Myc6xERE cells survived the selection. The experiment was repeated once more, selecting amplified cells with 15 mM G418 after having performed the 1 month treatment with 10 nM E2. The selection process took approximately 2.5 months and resistant cells for both MCF7/c-Myc 6xERE and MCF7/c-Myc cells were selected. No significant difference in colony count was observed between test and control cells.

In attempt to improve the selection strategy for cells with DNA amplification at the ectopic c-Myc replication origin, as outlined in Year-One Annual Report, I built a new construct featuring the following characteristics (Figure 2):

- <u>blasticidin resistance marker</u>. Cells with amplification at the ectopic DNA replication origin will be resistant to an increased drug concentration as compared to cells with no amplification because of the co-occurance of DNA amplification for the adjacent blasticidin resistance gene. The IC50 of blasticidin is much lower than G418 (approximately 2 μg/mL compared to 250 μg/mL, respectively), thus the usage of blasticidin should reduce costs for performing the amplification selection.
- <u>TagGFP2</u> (Evrogen). If DNA amplification occurs at the ectopic locus, a gain in TagGFP copy number will provide an increase in fluorescence signal in comparison to non-amplified cells. TagGFP2 will provide the opportunity for a faster and independent amplification screening strategy compared to drug selection.
- <u>The 2A peptide</u> is placed in between the two markers that will be thus encoded in a bicistronic mRNA. The 2A peptide ensures an equal expression level of the two markers (de Felipe *et al.*, 2006).
- The mouse PGK (phosphoglyceraldehyde kinase) promoter will drive a low expression level of the markers, thus avoiding toxic effects due to excessively high GFP levels in amplified cells and also restricting the increase of drug resistance in amplified cells.

Cloning of the construct *de novo* took roughly 5 months. An extensive period of cloning was required because each element (PGK promoter; blasticidin-2A; TagGFP2, the engineered c-Myc replication origin) was derived from different constructs and multiple intermediate steps were necessary to insert/remove restriction sites. Each intermediate cloning step was carefully validated by restriction digestions and sequencing. pTagGFP2 expression plasmid was purchased from Evrogene and TagGFP2 ORF was PCR amplified using a forward primer containing at its 5'-end a BamHI restriction site and the 54 bp 2A sequence, while the reverse primer contains at its 5'-end a BamHI restriction site. The PCR product was digested with BamHI and ligated into p101711-4 plasmid (kindly provided by Dr. Yamamoto Yutaka, MCB Department, Brown University). The resulting plasmid, called p101711-4/GFP carries the blasticidin-2A-TagGFP2 ORF, which was subsequently isolated following digestion with MscI. The mouse PGK promoter has been derived from the pSUPER.retro.puro plasmid (Oligoengine, kindly provided by Dr. Marco DeCecco, MCB department, Brown University). An EcoRI-AscI-EcoRI linker was ligated into EcoRI linearized pSUPER.retro.puro plasmid, and the modified pSUPER.retro.puro+EcoRI-AscI-EcoRI plasmid was subsequently digested with XbaI and NdeI, Klenow treated to create blunt ends, recircularized, and then NheI-linearized to integrate an oligonucleotide containing the Kozak sequence just downstream of the PGK promoter. The PGK promoter, complete with the Kozak sequence, was isolated from the plasmid by digestion with AscI. The final constructs (Figure 2) were prepared starting with the pFRT.Myc and pFRT.Myc_6xERE plasmids previously used to establish the recombinant MCF7 cell lines. An XbaI-AvrII-AscI-BamHI linker was integrated into the XbaI and BamHI digested plasmids, and subsequently he PGK promoter was integrated into the modified pFRT.Myc+XbaI-AvrII-AscI-BamHI and pFRT.Myc_6xERE+XbaI-AvrII-AscI-BamHI

constructs at the AscI site located upstream of the c-Myc replication origin. The resulting constructs were linearized with MscI to integrate the blasticidin-2A-TagGFP2 ORF downstream of the PGK promoter, thus obtaining the final pFRT.PGK-BlaGFP_Myc and pFRT.PGK-BlaGFP_Myc6xERE constructs.

The functional evaluation of marker genes within the *de novo* constructs were performed by assessing the expression of the TagGFP2 protein and the blasticidin resistance marker 24 hours post-transfection by RT-PCR (Figure 3). TagGFP2 expression was also analyzed using fluorescence microscopy (Figure 4).

MCF7 Flp-In host cell lines that were established and characterized in Year-One (MCF7/1B2, MCF7/2A2, and MCF7/3D2) have been co-transfected with the Flippase recombinase expression vector and either one of the FRT donor vectors (the new pFRT.PGK-BlaGFP_Myc or pFRT.PGK-BlaGFP_Myc6xERE constructs) in order to obtain the integration of the c-Myc origin at the donor FRT genomic site by homologous recombination. The FLP/FRT system establishes isogenic cell lines, thereby avoiding differences between the test and control cells due to positional effects. To control for transfection efficiency, parallel transfections were performed with pTagGFP2 plasmid, end expression of GFP checked after 24 hours (Figure 5). However, blasticidin selection proved inefficient at distinguishing cells with the integrated construct from cells that kept the construct as an episome. Indeed, after isolation and expansion of ~50 colonies from either MCF7/2C, or MCF7/1D, or MCF7/3B2 transfected with either of the 2 constructs, a diagnostic PCR failed in all but one clones to verify the integration of the vector at the genomic FRT site via homologous recombination (Figure 6). Nonetheless, PCR diagnostic for the presence of the blasticidin-2A-TagGFP2 ORF confirmed the presence of the vector in almost all the clones (Figure 7). To optimize the design of the constructs in order to solve this problem, several more cloning steps were subsequently performed. To have blasticidin to select exclusively for those cells in which the homologous recombination occurs at the genomic FRT site, both the PGK promoter and the ATG starting codon were removed from the blasticidin-2A-TagGFP2 ORF. Indeed, the blasticidin cassette is expressed only after successful integration through the SV40 promoter and an in frame ATG codon located upstream the genomic acceptor FRT site (Figure 8).

As proposed in Year-One Annual Report, during Year-Two I began work with a U2OS cell line that is stably transfected with a tetracycline-inducible vector to express ER-alpha. This cell line was kindly provided by Dr. Dale Leitman (Center for Obstetrics, Gynecology and Reproductive Sciences, UCSF). U2OS/ER-alpha cells have been tested for the inducible ER-alpha expression upon doxycycline stimulation by Western blot and the activation of estrogen cell response has been tested by qPCR by measuring change in mRNA expression level of known ER-alpha target genes upon treatment with 10 nM E2 (Figure 9).

In comparison to the MCF7 cell line, where control for the genomic integration site of the construct by the FRT/FLP system was necessary to avoid positional effects, the U2OS/ER-alpha cells allow for a random-integration-sites approach, as the same clone will be used as either the test or control cell line. Specifically, the control experiment will be performed in the absence of ER-alpha expression (no doxycyclin induction). To efficiently obtain cells stably carrying the engineered c-Myc replication

origin, I used the PiggyBac Transposon system (Figure 10): inverted repeat elements at the construct boundaries are recognized by the PiggyBac (PB) transposase, which mediates the integration of the construct into the cell genome. I modified the pFRT.PGK-BlaGFP_Myc6xERE construct designed for the MCF7 Flp-In system to adapt it to for the PiggyBac system: Minimal terminal repeats (235 bp at 3'-end and 310 bp at 5'-end) have been cloned at the construct boundaries (Li *et al.* 2001) (Figure 11). The cloning took about 2 months; difficulties were met in obtaining a successful ligation product. The sequence of the final construct was verified by sequencing.

To find the optimal transfection conditions, trial experiments were performed with different amount of construct (pPB.PGK-BlaGFP_Myc6xERE) and PB Transposase expression vector (pCMVhyPBase). Transposition efficiency was determined by colony count after blasticidin selection and crystal violet staining (Figure 12). Upon identification of the optimal transfection condition to isolate single integrant clones, $1x10^5$ U2OS/ER cells have been co-transfected with 10 ng PB Transposase, and either 5 ng or 10 ng of the pPB.PGK-BlaGFP_Myc6xERE. To enhance the likelihood of selection of independent integration events, each transfection condition was performed in duplicates. A fraction of the cells have been used to determine transposition efficiency (Figure 13). I isolated and expanded 25 clones and Southern Blot was performed to identify those clones that carry a single copy of the construct (Figure 14).

Some clones carrying a single copy of the construct were used to perform FACS analysis to determine the baseline TagGFP2 signal before DNA amplification at the ectopic Myc replication origin (Figure 15). Moreover, I compared GFP signal of cells carrying 1 copy, 2 copies (clone U2OS/i2A1) or 4 copies (clone U2OS/i1A2) of the fluorescent marker (Fig 15) in an effort to perform a preliminary tests to assess the capability of the FACS analysis to distinguish cells in which DNA amplification occurred at the ectopic Myc origin. To note anyway that this is only an approximate evaluation, since marker expression level is influenced by the surrounding genomic sequence at the integration site.

- **Subtask 2d (month 18-19): Real time PCR to quantify amplification at the c-Myc locus.**

During Year-One, in anticipation of this subtask, I performed qPCR to assess the DNA copy number at the ectopic c-Myc origin, after E2 treatment and found an average of 12-fold amplification in high dose G418 selected cells. Encouraged by this exciting preliminary result, I measured DNA copy number at the ectopic c-Myc locus for MCF7/c-Myc and MCF7/c-Myc6xERE cells selected with high dose of G418 in two subsequent experiments, which were carried out during Year-Two (see subtask 2c). In the second experiment, I selected for DNA amplification using 20 mM G418, a concentration previously known to be well tolerated by cells with DNA amplification at the ectopic locus. However, only few colonies of MCF7/c-Myc control cells were recovered after high dose G418 selection, and qPCR showed an average of ~2-fold amplification at the ectopic c-Myc origin (Figure 16). From my third experiment, I obtained both MCF7/c-Myc and MCF7/c-Myc6xERE colonies resistant to high dose G418, but qPCR show no DNA amplification at the ectopic c-Myc origin for both the test and control selected cells (Figure 17).

Task Three – Can Estrogen Induce DNA Amplification at the Other Genomic Loci? (month 20-23)

Due to extensive efforts directed towards the cloning to obtain the revised reporter construct and the establishment of the new U2OS cell model, the assessment of amplification at the ectopic locus (Task Two) using the new reporter vector has been delayed. Task Three of the approved SOW will be thus addressed when reproducible evidence of DNA amplification at the engineered ectopic locus will be obtained.

Task Four – Does ER-alpha Directly Interact With the Replication Machinery? (months 23-32)

- **Subtask 4a (months 23-25): Preparation and cloning of the FLAG-ER-alpha and FLAG-c-Myc constructs into a tetracycline inducible response vector.**

Task Two constitutes the essential starting element for testing the main hypothesis of estrogen inducing re-replication when acting close to DNA replication origin, and more efforts then foreseen have been required to build the reporter construct. As a result, this subtask has not been addressed. However, a plasmid expressing a FLAG-Orc2 protein has been requested and already kindly provided by Dr. Melvin DePamphilis (NIH, Bethesda, MD). To investigate the molecular interaction between the replication machinery and the ER-alpha, I can indeed take advantage of the U2OS/ER-alpha cells that is already in use. A FLAG-tagged ORC2, in conjunction with an induced expression of the ER-alpha are likely to be optimal conditions for a co-IP experiment. A stock of the plasmid has been prepared and the expression of the Flag-Orc2 protein has been assessed by Western blot in transfected MCF7 cells.

KEY RESEARCH ACCOMPLISHMENTS

- Establishment of a new construct to be used in Flp-In host cells to establish recombinant clones via the FLP/FRT system;
- Construction of a modified construct to be used for the PiggyBac transposition system of gene delivery, which efficiently generates stable integrant clones;
- Determination of PiggyBac transposition condition for U2OS cells;
- Establishment of a panel of recombinant U2OS/ER-alpha_PGK-BlaGFP_Myc6xERE cells.

REPORTABLE OUTCOMES

i.   Manuscripts, abstracts, presentations: None

ii.   Licenses applied for and/or issued: None

iii.   Degrees obtained that are supported by this award: None

iv.   Development of cell lines, tissue or serum repositories: U2OS recombinant clones carrying an optimize construct for DNA amplification selection.

v.   Informatics such as databases and animal models: None

vi.   Funding applied for based on work supported by this award: None

vii.   Employment or research opportunities applied for and/or received based on experience/training supported by this award: None

CONCLUSION

During Year-Two, I continued on work carried out in Year-One, repeating the estrogen treatment and drug selection for MCF7/Myc and MCF7/Myc6xERE with amplification at the ectopic c-Myc origin. However, I was unable to repeat the promising preliminary results of ~12-fold amplification obtained during Year-One. In fact, in one case the high dose drug selection did not enrich for cells with DNA amplification, suggesting that a more careful drug titration is required to avoid selection of false positives. Moreover, the assessment of an average of ~2-fold amplification for the MCF7/Myc control cell line suggests that a low level amplification at the ectopic c-Myc origin is possible even in the absence of the ER-alpha binding site. Nonetheless, a specific role of ER-alpha in triggering DNA amplification could be assessed on the different level of amplification.

In an attempt to ameliorate the experimental design and to shed light on the controversial results obtained in this first round of experiments, I designed and constructed a new vector comprising of two selection markers that allow two independent selection strategies for cells with DNA amplification at the ectopic c-Myc origin: increased drug resistance (>1 copy drug resistance gene) and increased GFP signal (>1 copy TagGFP2).

As a second system to test estrogen inducing DNA amplification when acting close to a DNA replication origin, a U2OS cell line conditionally expressing ER-alpha has been used to establish recombinant cell lines carrying the engineered c-Myc origin. The construct designed for the FLP/FRT system has been adapted to obtain recombinant U2OS/ER-alpha_ PGK-BlaGFP_Myc6xERE cells containing a single copy of the integrated construct via the PggyBac transposition system. The PiggyBac transposition system has proven very efficient in generating recombinant cells (3×10^{-3}). The assessment of the ER-alpha expression and the TagGFP2 fluorescent signal by FACS support the suitability of these recombinant cells for testing and selecting for the presence of DNA amplification at the ectopic replication origin after estrogen treatment.

The importance of this project lies in the elucidation of a possible mechanism for initiation of DNA amplification, which is potential clinical importance due to the prevalence of DNA amplification in cancer (e.g. Myc oncogene is often amplified in multiple cancers and its copy number state correlates with disease prognosis).

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Yusa K, Zhou L, Li MA, Bradley A, Craig NL. (2011) A hyperactive piggyBac transposase for mammalian applications. Proc Natl Acad Sci U S A. 108(4): 1531-6.
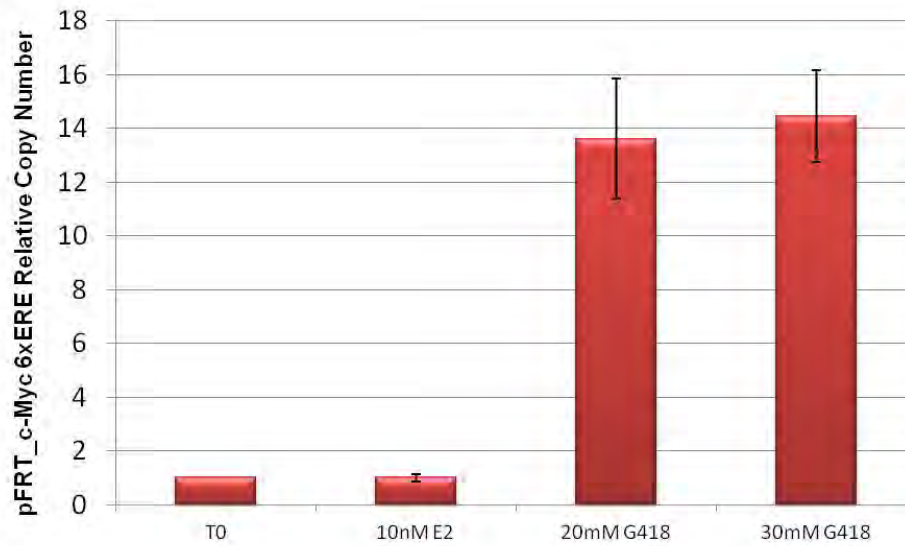
Figure 1. Evaluation of DNA Amplification at the Ectopic c-Myc Origin.
MCF7/FRT c-Myc 6xERE were treated for 1 month with 10 nM E2 and then
treated with 20 mM G418 and 30 mM G418. Genomic DNA was isolated from
cells collected at time zero (T0), after the steroid hormone treatment (10 nM
E2), and from cells survived to the high dose G418 selection (20 mM G418
and 30 mM G418). The vector specific sequence was normalized to TBP
(TATA Binding Protein).

A FRT

| PGK promoter | BlaR | 2A | TagGFP2 | | c-Myc origin |

B FRT

| PGK promoter | BlaR | 2A | TagGFP2 | | c-Myc origin | 6xERE |
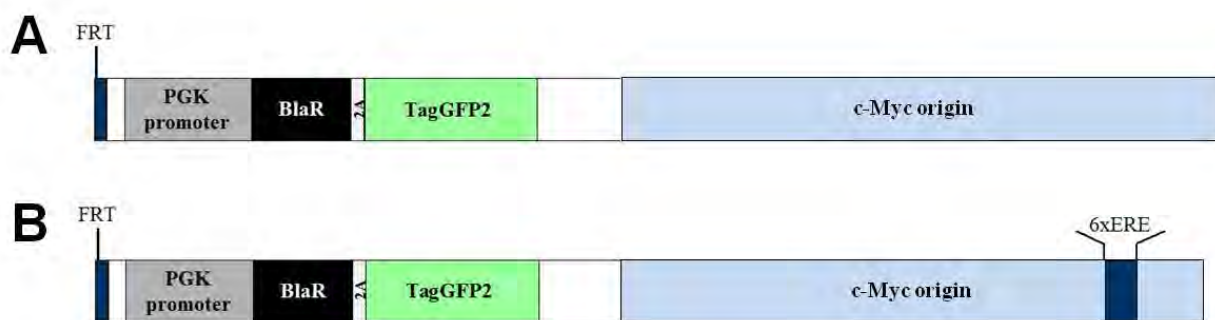
Figure 2. Scheme of A) pFRT/c-Myc_PGK_Bla2AGFP and B) pFRT/c-Myc 6xERE_PGK_Bla2AGFP Constructs. The modified constructs to be integrated in the newly established MCF7 Flp-In cells will have i) a mouse PGK promoter for a low level transcription, ii) a blasticidin resistance marker for a drug-based selection of cells after 17β-estradiol treatment, iii) TagGFP2 (Evrogen) for fluorescence-based selection, iv) the 2A peptide that ensures an equal transcription level of the two markers, which are expressed as a bicistronic transcript.
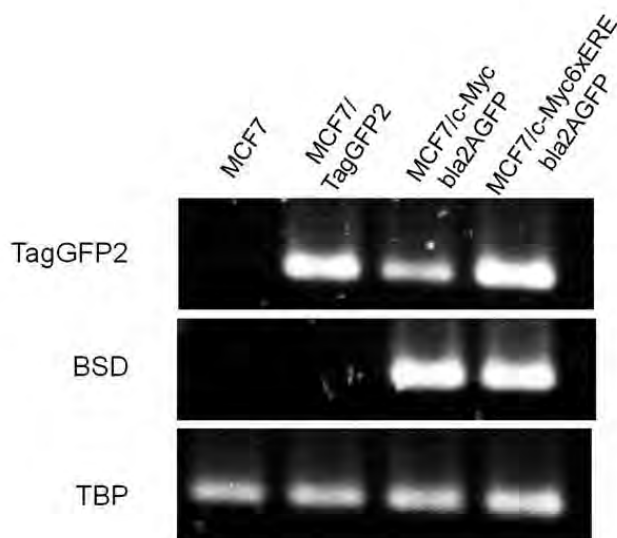
Fig. 3 Expression of Selection Markers from *de novo* Construct. Expression of blasticidin resistance marker and TagGFP2 upon cell transfection with either pFRT.PGK-BlaGFP_Myc or pFRT.PGK-BlaGFP_Myc6xERE have been assessed by RT-PCR. MCF7: negative control; MCF7/TagGFP2: cell transfected with pTagGFP2 plasmid as positive control for GFP expression; BSD: blasticidin resistance gene; TBP: TATA Binding Protein as PCR control.
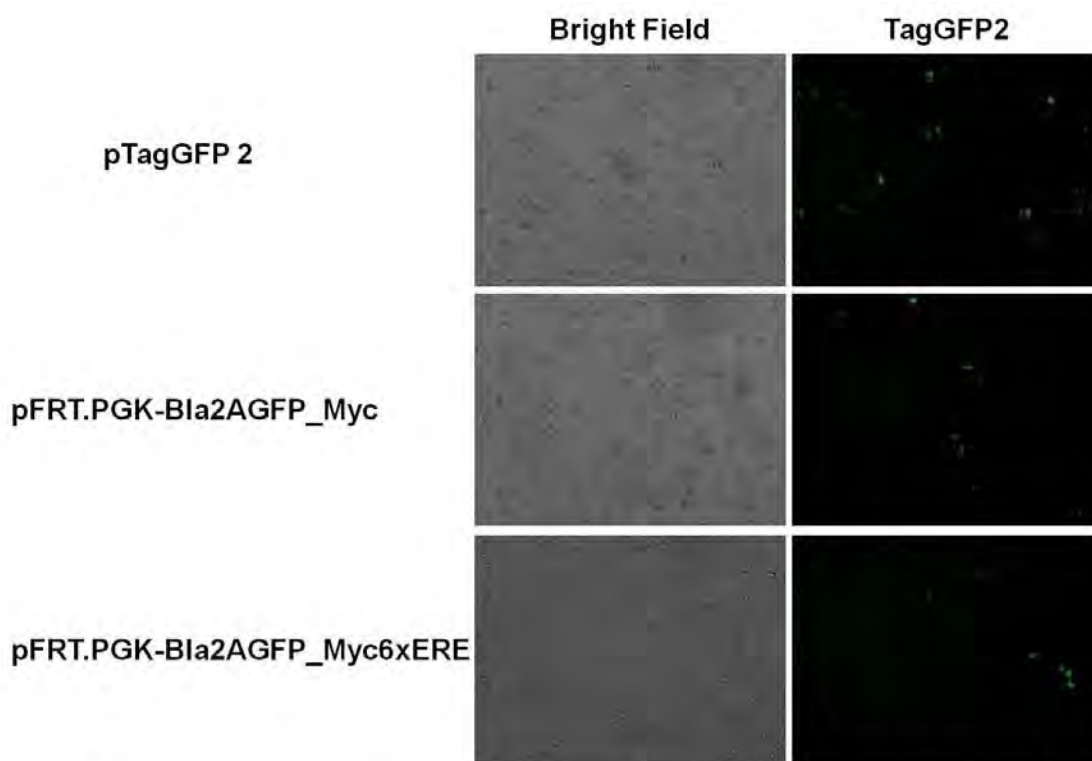
Fig 4. TagGFP2 Expression Upon pFRT.PGK-BlaGFP_Myc and pFRT.PGK-BlaGFP_Myc6xERE Transfection. MCF7 have been transiently transfected with either pTagGFP2 (positive control), pFRT.PGK-Bla2AGFP_Myc or pFRT.PGK-Bla2AGFP_Myc6xERE. TagGFP2 expression has been successfully detected in cells transfected with *the novo* constructs.
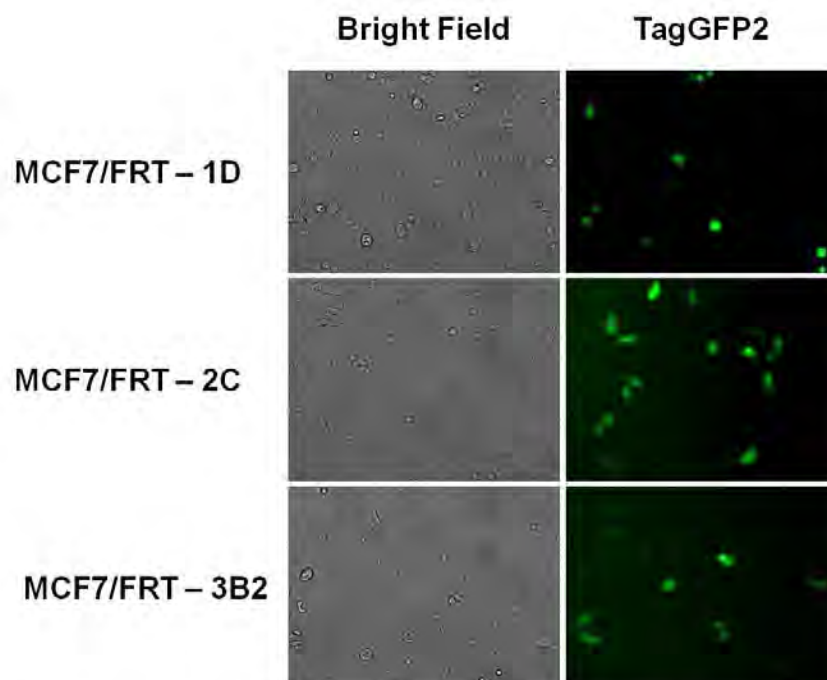
Fig 5. Establishment of Stable Recombinant MCF7/PGK-BlaGFP_Myc and MCF7/PGK-BlaGFP_Myc6xERE Cells. MCF7 Flp-In clones established and characterized during Year-One have been co-transfected with either pFRT.PGK-Bla2AGFP_Myc or pFRT.PGK-Bla2AGFP_Myc6xERE and the Flippase recombinase expression plasmid. Transfection efficiency have been assessed using pTagGFP2 and monitoring GFP expression at 24 hours post transfection at the fluorescent microscope. Transfection efficiency has been estimated to be about 30-50%.
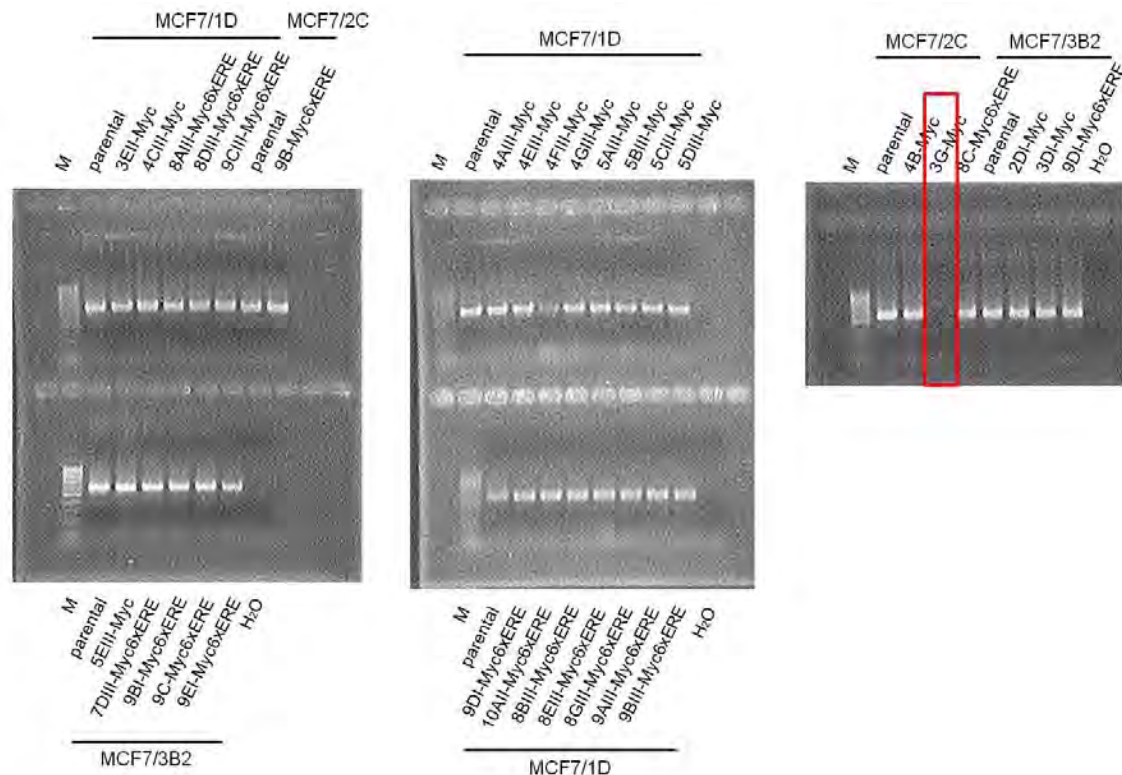
Fig 6. Diagnostic PCR to Evaluate Construct Integration at the Genomic FRT Site in Flp-In acceptor cells. Flp-In cell lines MCF7/1D, MCF7/2D, and MCF7/3B2 were transfected with either pFRT.PGK-Bla2AGFP_Myc or pFRT.PGK-Bla2AGFP_Myc6xERE vectors. A successful homologous recombination determines the integration of the ~7 kb donor construct at the genomic FRT site in Flp-In acceptor cells. A diagnostic PCR that amplifies the acceptor FRT site have been done on genomic DNA isolated from 50 blasticidin resistant clones. A PCR product is diagnostic for parental Flp-In cells and cells where recombination did not specifically occur at the FRT site. A successful recombination spaces primers by ~ 8 kb and no PCR product will be obtained. This analysis revealed that a successful recombination occurred only once (clone 2C-3G-Myc) out of 50 clones examined. M: 100 bp DNA ladder. H₂O: water as negative control sample; parental: parental Flp-In cell lines as PCR positive control.

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Fig 7. Diagnostic PCR to Evaluate the Presence of either the pFRT.PGK-BlaGFP_Myc or pFRT.PGK-BlaGFP_Myc6xERE Construct. Blasticidin-GFP ORF have been PCR amplified from genomic DNA of a subset of blasticidin resistant clones to assess for the presence of the transfected vector. Parental cell lines and water have been used as negative controls. Even if a successful recombination at the genomic FRT site occurred in none but one of the isolated clones, this PCR shows that in the vector is however present in almost all of them.

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MCF7 Flp-In Acceptor cell line :
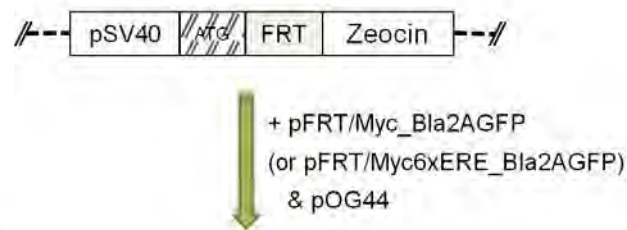
Recombinant cell line:

Fig 8. Schematic Representation of the Latest Version of the Construct to be Used in MCF7 Flp-In cells to Assess DNA Amplification at the Ectopic Replication Origin. To prevent selection of cells that retain the construct as an episome, the revised vectors containing the 2.4 kb c-Myc origin (in the figures outline as red boxed) do not have a promoter. The blasticidin-2A-TagGFP2 ORF will be transcribed from the SV40 promoter located upstream of the acceptor FRT sequence integrated into the genome of the Flp-In host MCF7 cells. Moreover, the ATG start codon is present only upstream of the acceptor FRT sequence, and will be available to the blasticidin-2A-TagGFP2 ORF only when a successful homologous recombination occur at the genomic FRT site.
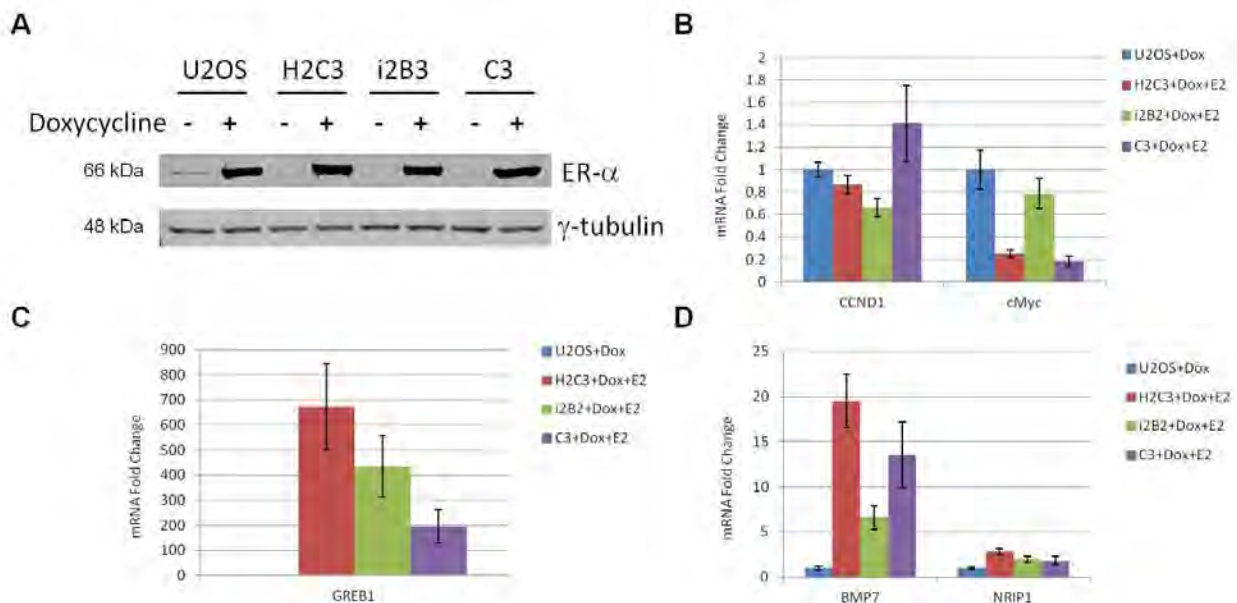
Fig 9. Functional ER-alpha Pathway in Recombinant U2OS/ER-alpha_PGK-BlaGFP_Myc6xERE. A) Activation of ER-alpha expression upon doxycycline induction has been assessed at 24 hour by Western blot for 3 independent recombinant U2OS/ER-alpha_PGK-BlaGFP_Myc6xERE clones, namely H2C3, i2B3, and C3. Parental U2OS/ER-alpha cells has been used as positive control. While the heterogeneous parental cell line shows some expression of ER-alpha even in absence of doxycycline, the 3 PGK-BlaGFP_Myc6xERE clones show a good control on the induction of ER-alpha expression. B) C) D) qPCR evaluation of mRNA fold change for known ER-alpha target genes upon doxycycline induction. As expected, ER-alpha is active in U2OS/ER-alpha clones and modulates the expression of its target genes. Parental U2OS/ER-alpha cells without doxycycline treatment have been used as reference sample. Expression levels have been normalized to GAPDH mRNA.
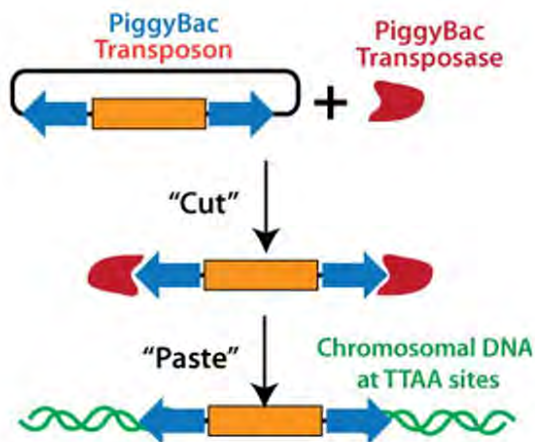
23

Fig 10. Schematic Representation of the PiggyBac Transposon System. PiggyBac Transposase recognizes transposon-specific inverted terminal repeat sequences (ITRs) located at both ends of the transposon vector and with a "cut and paste" mechanism the sequence is integrated into TTAA chromosomal sites. The PiggyBac transposase expression vector lacks selection marker and it is diluted during cell division, thus preventing the excision of the integrated cassette.

Fig 11. Schematic Representation of the Construct for the PiggyBAC System. In the recent years the PiggyBac system has been proved to be an efficient tool for gene delivery in mammalian cells. The pFRT.PGK-Bla2AGFP_Myc6xERE construct has been implemented with the PiggyBac-specific inverted terminal repeats (5' ITR and 3 'ITR) to use the PiggyBac transposition system to generate the recombinant U2OS/ER-alpha_PGK-BlaGFP_Myc6xERE. Upon co-transfection of the vector and the PiggyBac transposase expression plasmid, the engineered c-Myc replication origin and the selection markers will be randomly integrated at chromosomal AATT sites.
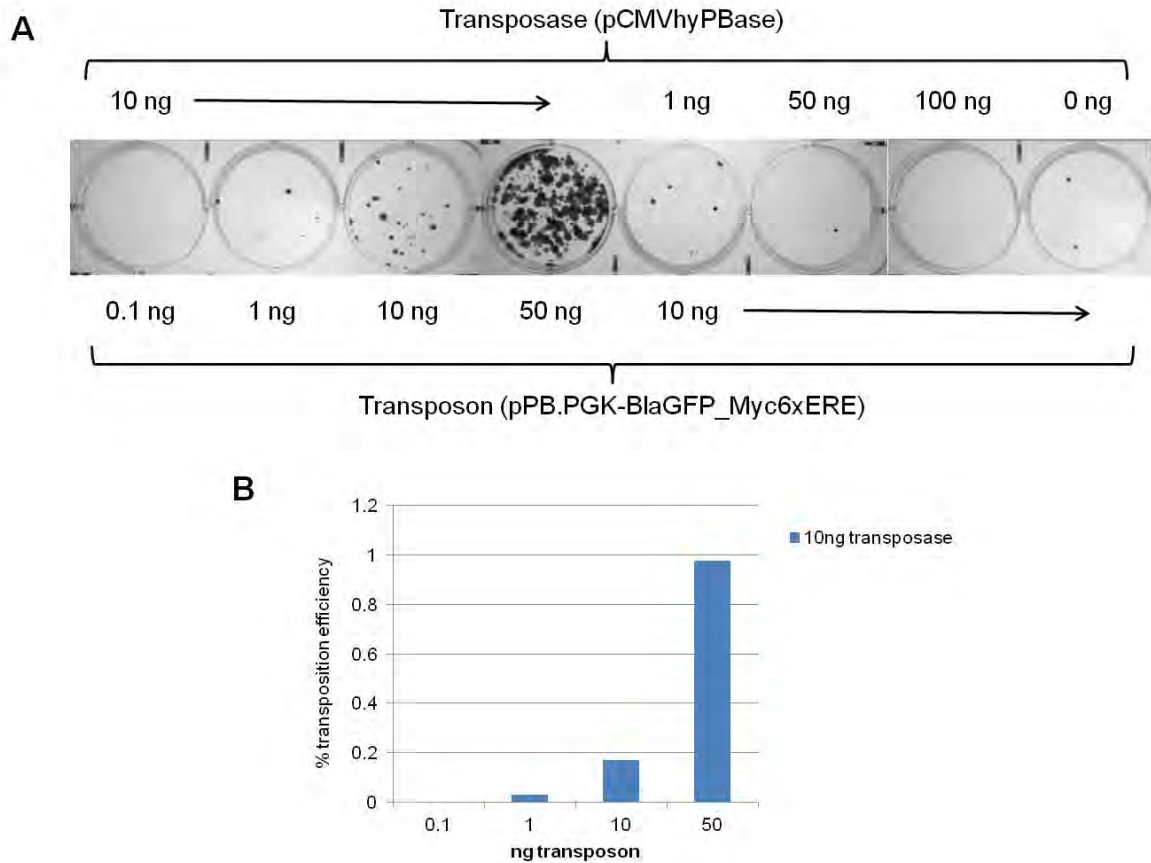
A

Transposase (pCMVhyPBase)

10 ng ⟶   1 ng   50 ng   100 ng   0 ng

0.1 ng   1 ng   10 ng   50 ng   10 ng ⟶

Transposon (pPB.PGK-BlaGFP_Myc6xERE)

B

Fig 12. Determination of the Transposition Efficiency with PiggyBac System. U2OS/ER-alpha cells have been transfected with different amounts of pPB.PGK-BlaGFP_Myc6xERE and pCMVhyPBase to find the conditions required for generating the stable recombinant clones. After transfection and blasticidin selection, resistant colonies were stained with crystal violet (A) and counted to determined transposition efficiency (B). The PiggyBac system has proved to be very efficient and 1-10 ng of pPB.PGK-BlaGFP_Myc6xERE with 10 ng pCMVhyPBase have been determined to be the optimal conditions.
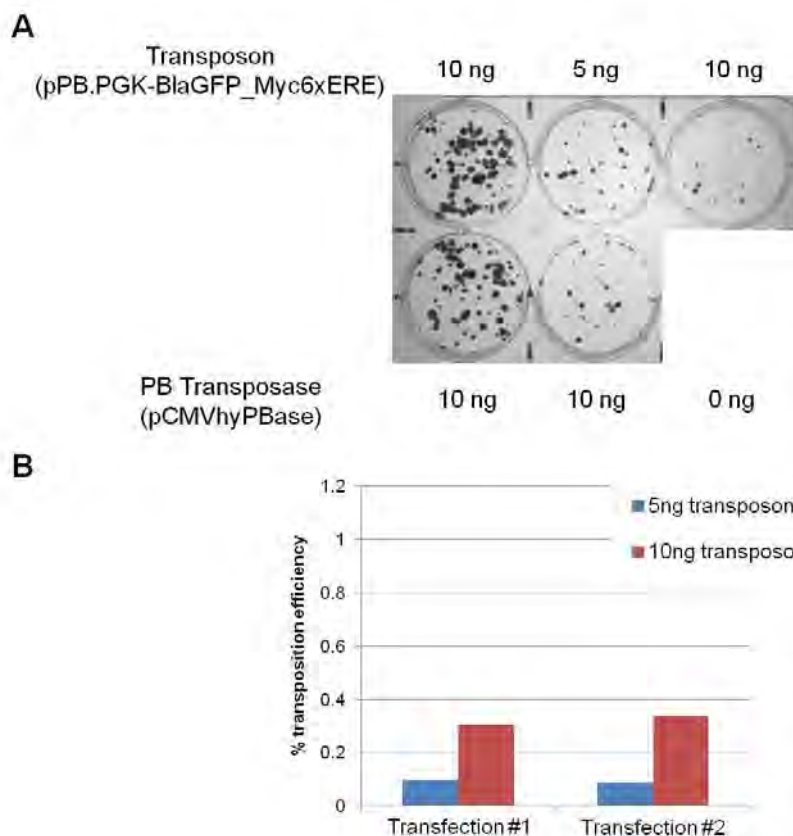
A

Transposon
(pPB.PGK-BlaGFP_Myc6xERE)   10 ng   5 ng   10 ng

PB Transposase
(pCMVhyPBase)   10 ng   10 ng   0 ng

B

Fig 13. Establishment of Stable Recombinant U2OS/ER-alpha_PGK-BlaGFP_Myc6xERE Clones. Based on previous transposition efficiency results, U2OS/ER-alpha cells have been co-transfected with either 5 ng or 10 ng pPB.PGK-BlaGFP_Myc6xERE and 10 ng pCMVhyPBase. These conditions maximize the probability to obtain single integrant clones. After 24 hr from transfection cells were diluted, re-plated and selected with 4 μg/mL blasticidin for either crystal violet staining (A) and colony count to determine transposition efficiency (B) or for single clone isolation. After blasticidin selection 25 independent clones were isolated.
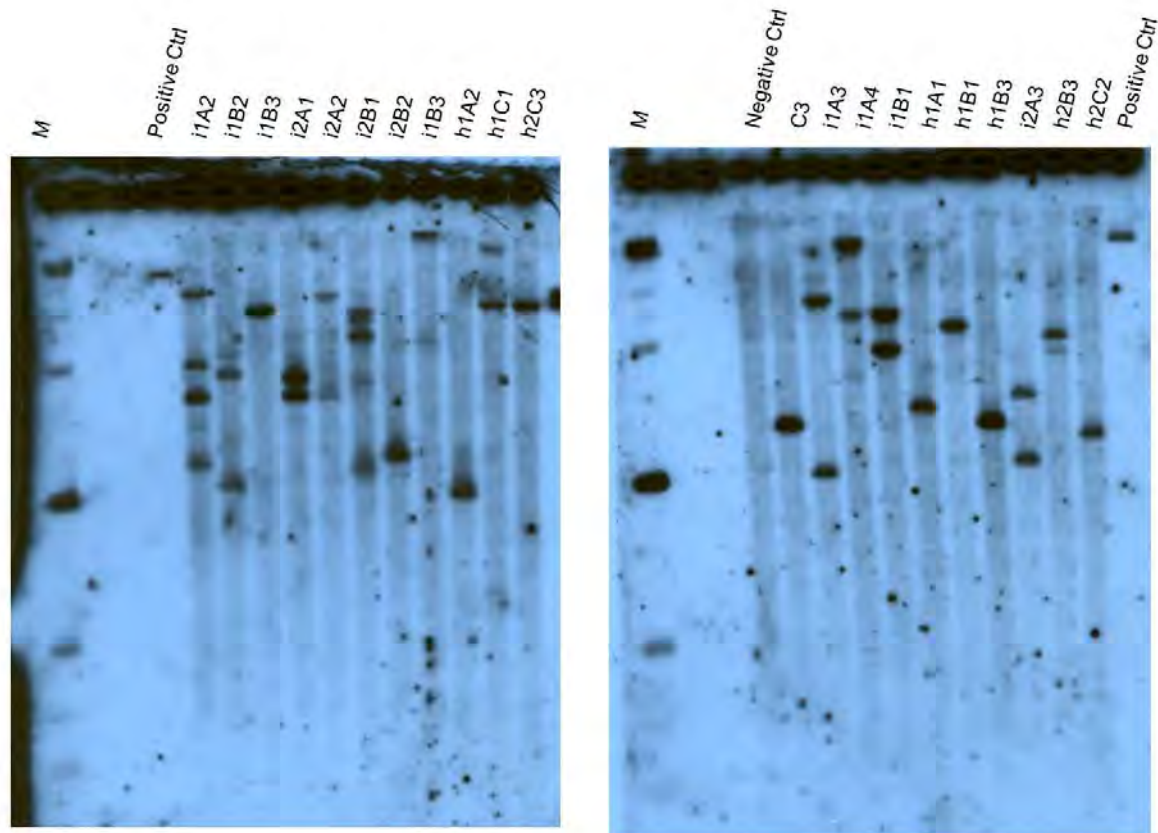
Fig 14. Southern Blot of U2OS/ER-alpha_PGK-BlaGFP_Myc6xERE Clones. Upon cell transfection and blasticidin selection, 25 independent clones were isolated and Southern blot performed to identify clones with a single copy of the pPB.PGK-BlaGFP_Myc6xERE vector. Results show that the transfection condition used allowed to integrate one copy of the vector per genome in half of the cases (11 out of the 21 clones analyzed). Clones named hXXX derive from transfections done with 5 ng of the vector, while the iXXX serie derives from transfections with 10 ng vector. M: 1 kb DNA ladder; Positive Ctrl: 10 ng linearized pPB.PGK-BlaGFP_Myc6xERE vector; Negative Ctrl: HindIII digested genomic DNA from non transfected U2OS/ER-alpha cells.
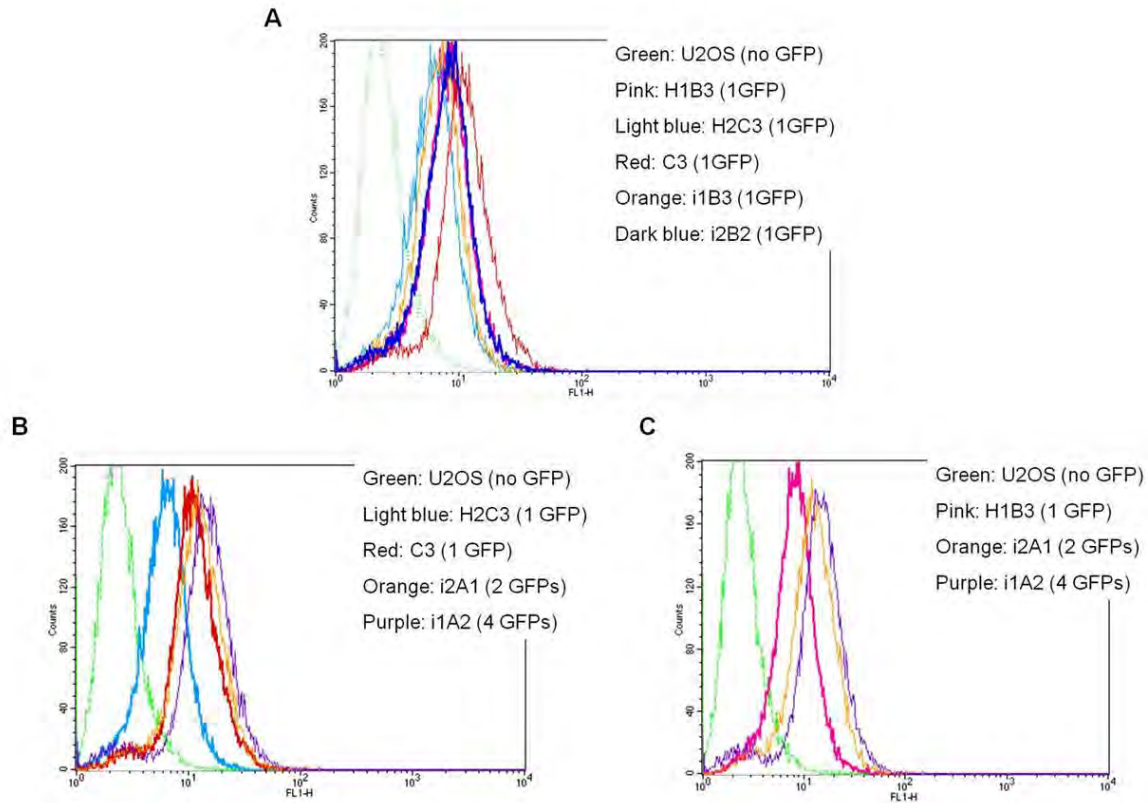
Fig 15. FACS Analysis of Basal GFP Expression in U2OS/ER-alpha_PGK-BlaGFP_Myc6xERE Clones . A) Basal expression of GFP was evaluated by FACS for 5 independent U2OS/ER-alpha_PGK-BlaGFP_Myc6xERE clones that carry a single copy of the vector. Parental U2OS/ER-alpha have been used as negative control. The 5 clones identify 3 different level of GFP expression: low (H2C3 and i1B3), medium (H1B3 and i2B2), and high (C3). B) and C) Comparative approximated analysis of clones that contain 1 copy (H2C3, H1B3 and C3), 2 copies (i2A1), and 4 copies (i1A2) of the integrated vector. GFP signal profiles show that at the increase of GFP copy number increase the intensity of the total fluorescent signal.
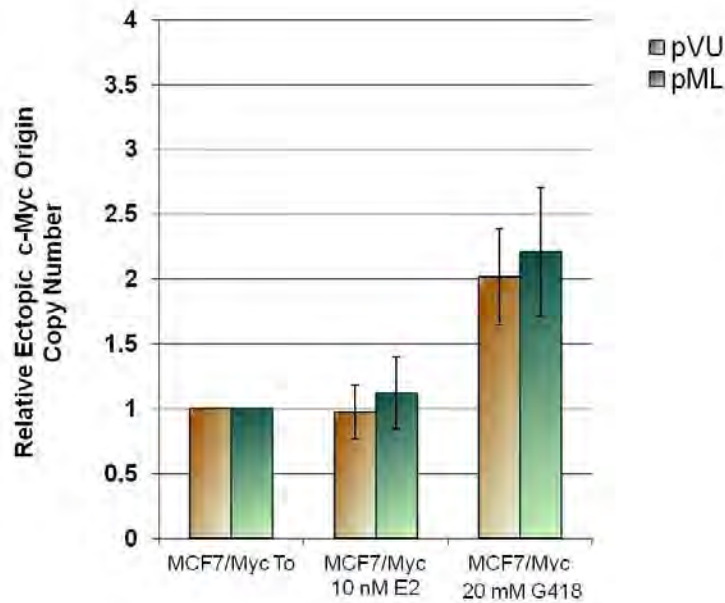
Fig 16. Real Time PCR Assessment of DNA Amplification – Exp#2. After 10 nM E2 treatment and subsequent G418 selection, only MCF7/Myc resistant cells have been isolated. Copy number assessment at the 3' (pML probe) and 5' (pUV probe) of the ectopic c-Myc locus suggests an average of 2-fold amplification at this genomic site. MCF7/Myc To: before E2 treatment; MCF7/Myc 10 nM E2: after E2 treatment and before G418 selection; MCF7/Myc 20 mM G418: cells resistant to 20 mM G418 after E2 treatment. The vector specific sequence was normalized to TBP (TATA Binding Protein).
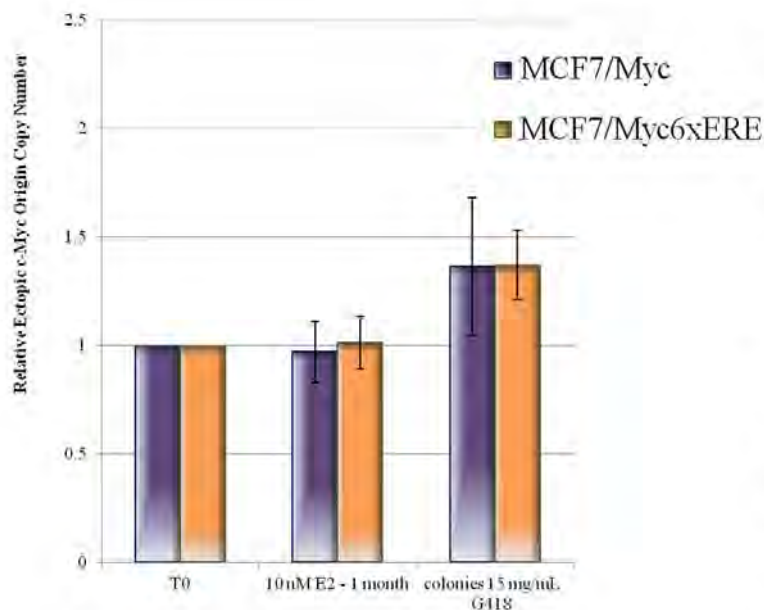

Fig 17. Real Time PCR Assessment for DNA Amplification – Exp#3. After 10 nM E2 treatment and subsequent G418 selection, resistant MCF7/Myc and MCF7/Myc6xERE cells have been isolated and copy number assessment at the ectopic c-Myc locus performed (vector specific probe: pML). Data show absence of significant amplification for both the cell lines. T0: before E2 treatment; 10 nM E2 - 1 month: after E2 treatment and before G418 selection; MCF7/Myc 15 mM G418: cells resistant to 15 mM G418 after E2 treatment. The vector specific sequence was normalized to TBP (TATA Binding Protein).

SUPPORTING DATA

None